

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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JUN 19 2002

In re application of

Grahn HÅKANSSON et al.

Serial No. 09/623,562

GROUP 1651

Filed November 16, 2000

Examiner S. Saucier

TECH CENTER 1600/2900

AGENT

Declaration of Maria Isaksson

I, Maria Isaksson, a Swedish citizen residing at Överboda 202, 905 88 Umeå, hereby declare as follows:

I have a Masters of Science in Biomedicine in 2001. I was employed by Essum AB, Umeå, Sweden in 2001 as a project coordinator and leader, but I have been working in the field of the present investigation utilizing the methods described herein since 2000. I have also attended a basic course in clinical trials at the Swedish Academy of Pharmaceutical Sciences in 2002.

I have been asked by Ms. Ann Samuelsson, SCA Hygiene Products AB, Gothenburg, Sweden to perform tests to compare the performance of the two *Lactobacillus plantarum* strains mentioned here below in order to compare their performance in being transferred to and maintained in the urogenital area and their ability to inhibit growth of and kill common pathogenic urogenital bacteria. The tested *Lactobacillus plantarum* strains were:

Lactobacillus plantarum 931

Deposit number: DSM11918

Described in the application US 09/623,562

Assignee: SCA Hygiene Products AB

Strain ordered from: Deutsche Sammlung von Microorganismen und Zellkulturen

Mascheroder Weg 1b
D-38124 Braunschweig, Germany

Lactobacillus plantarum ATCC 8014

Deposite number: ATCC 8014

Described in the patent US 5,705,160

Strain ordered from: American Type Culture collection
12301 Parklawn Drive
Rockville, Maryland 20852, USA

The enclosed experimental results illustrate the advantages of using *Lactobacillus plantarum* 931, hereafter called LB931, over another *L. plantarum* strain, namely *L. plantarum* ATCC 8014, hereafter called ATCC 8014, as a probiotic bacterium provided on hygiene products. The performed experiments illustrate differences in transfer rates to and maintenance levels in the urogenital area and the ability of inhibiting growth of and killing pathogenic urovaginal bacteria between the two tested *Lactobacillus plantarum* strains.

Difference in transfer rates and maintenance levels of LB931 and ATCC 8014, provided via panty liners, in urethra and perineum areas

LB931 and ATCC 8014 are grown on MRS agar plates for two days at +37°C in CO₂ (5%). Then one colony of each strain is inoculated into 5 ml of MRS broth (start tube) and incubated in the same way as above, but just over night. 50 ul from the start tube is put into 5 ml MRS broth, pre heated to +37°, and is incubated at +37°C for 8 hours. In the afternoon 500 ul is put into 50 ml pre heated MRS broth and is incubated at +37°C for 16 hours.

This suspension is centrifuged for 20 min at 3000 x g at +4°C, and the pellet is washed three times in 10-20 ml NaCl (0,9%). After washing, the pellet is resuspended in 2,5 ml NaCl (0,9%) + 2,5 ml skimmilk, thereby concentrating the sample 10 x. This suspension is transferred to an actuator vial. Each 10 + 1 (test) panty-liners are sprayed

with about 150 mg bacteria suspension. The panty-liners are dried for 6 h in room temperature before being packaged one by one into mini-grip bags.

Control of colony forming units (CFU) of the test panty liner: 50 ml of NaCl (0,9%) is poured over the panty liner in a stomacher bag that is run at high speed for 3 min. The fluid is diluted and 100 ul is spread on MRS agar plates. The number of CFU/panty liner for ATCC 8014 was 3×10^9 and for LB931: 1×10^9 .

Voluntary women take a time zero sample (perineum and urethra) before wearing a panty liner supplied with either of the *L. plantarum* strains for 5 hours. The panty-liner is removed and sample 2 is taken. After 24 hours (from the time zero sample) sample 3 is taken.

Transfer is defined as % of total women that after 24 hours still carry bacteria.

Results:

As shown in fig. 1, more of the women who had used panty liners with LB931 still harbored the LB931 bacteria in the perineum 24 hours the time zero sample was taken (92% still harbored the bacteria). In comparison, of the women who had used panty liners with ATCC 8014 only 50% harbored the tested bacterial strain at the same time point in the perineum (fig. 2).

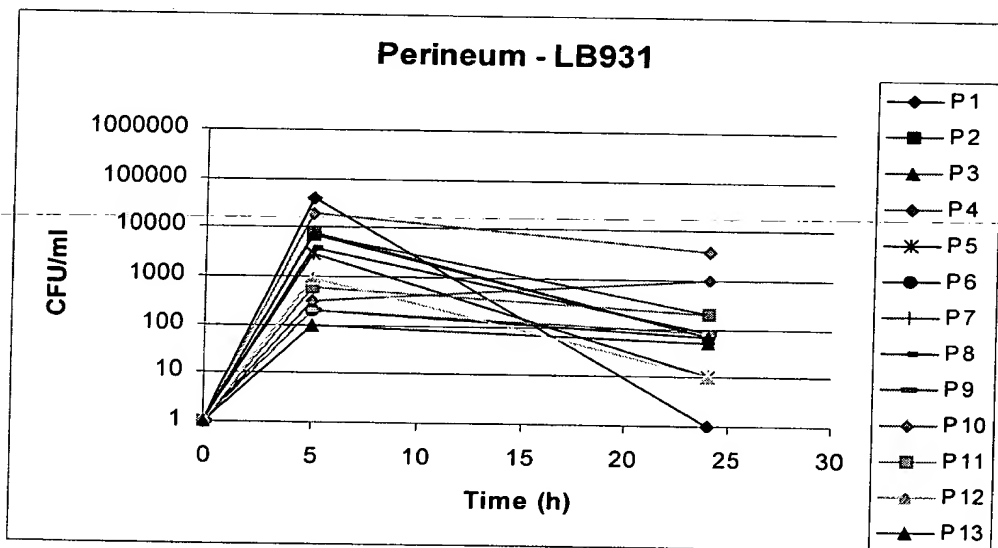


Fig. 1. Transfer and maintenance of *L. plantarum* 931 (LB931) in the perineum.

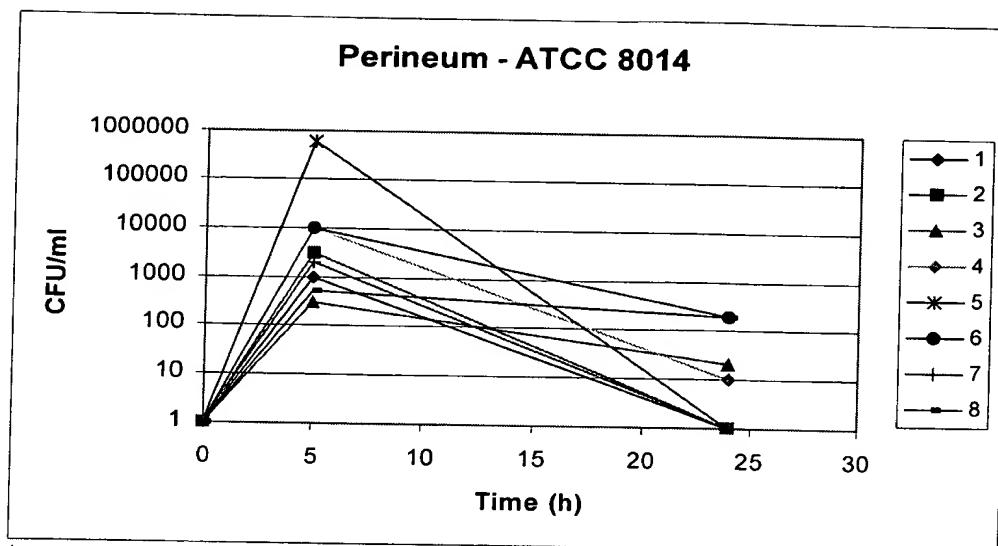


Fig. 2. Transfer and maintenance of *L. plantarum* ATCC 8014 in the perineum.

A similar difference was seen when the number of bacteria that still were present in the urethra was determined 24 hours after the time zero sample was taken. In this case 62% of the women who had used panty liners with LB931 harbored the bacteria (fig. 3), compared to 25% of the women who had used panty liners with ATCC 8014 (fig. 4).

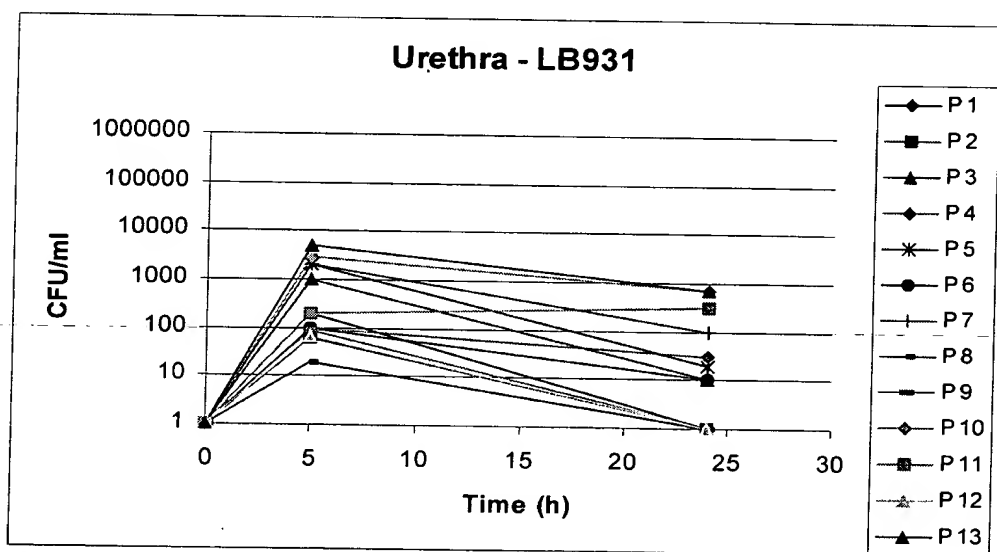


Fig. 3. Transfer and maintenance of *L. plantarum* 931 (LB931) in the urethra.

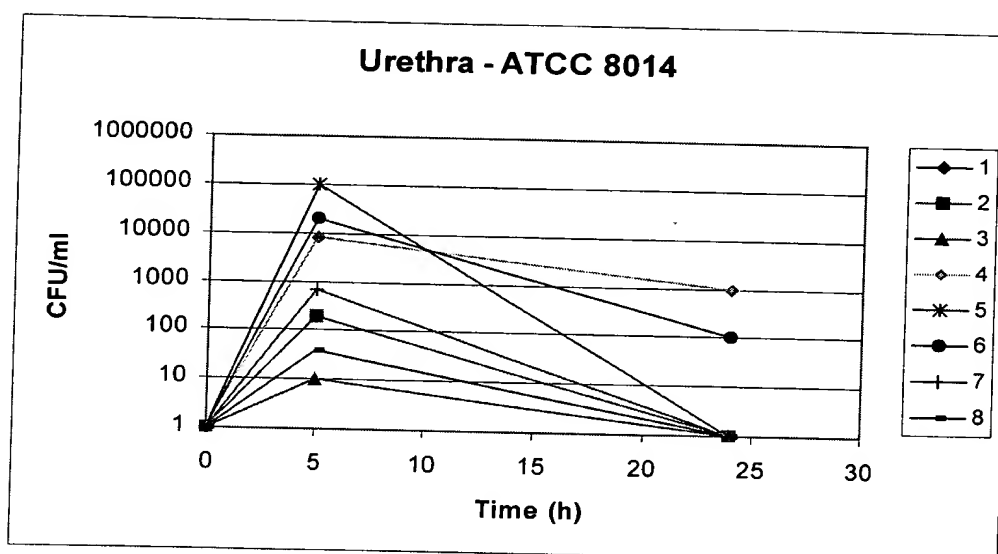


Fig. 4. Transfer and maintenance of *L. plantarum* ATCC 8014 in the urethra.

Comparison of ability of growth inhibition and killing of pathogenic bacteria between LB931 and ATCC8014

LB931 and ATCC 8014 are grown as described above, but to a final volume of 100 ml each. The suspension is centrifuged and resuspended in 10 ml of PBS, in a Falcon tube (size 50 ml). The tube is incubated at +37°C for 5 hours. Thereafter the suspension is centrifuged and the supernatant (PBS sup) collected, containing inhibiting substances secreted by the lactic acid bacteria. The PBS sup is sterile filtered through a 45 um and a 22 um filter consecutively. The PBS sup is stored at -20° until use. For controls PBS buffer, pH adjusted (pH 5,1) and unadjusted (pH 7,3), are used.

The ability of the two tested *Lactobacillus plantarum* strains, LB931 and ATCC 8014, to inhibit growth of common urogenital pathogens was tested. The tested pathogens were *Escherichia coli*, *Enterococcus*, *Staphylococcus aureus* and Group B *Streptococcus* (GBS). These bacteria were grown in TH broth and diluted 1:100 before 10 ul are inoculated into 1 ml of PBS sup. The numbers of pathogens is determined for the tested pathogenic strains at 0, 4, 6 and 24 hours.

Results:

As can be seen in figs 5-8 LB931 is superior over ATCC 8014 in inhibiting growth and actually killing pathogenic bacteria. In the case of *Escherichia coli*, *Enterococcus* and *Staphylococcus aureus* the growth of these pathogenic strains is not, or only marginally, inhibited by the ATCC 8014 strain, while the pathogenic bacteria are completely abolished when placed in the supernatant of LB931. In the case of Group B *Streptococcus* (GBS), LB931 supernatant kills all the bacteria within 4 hours, while ATCC8014 requires 24 hours to do the same.

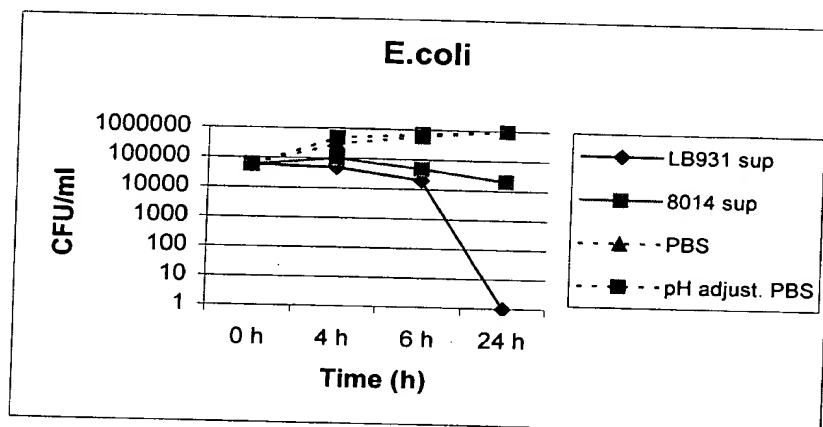


Fig. 5. Inhibition of *E. coli*.

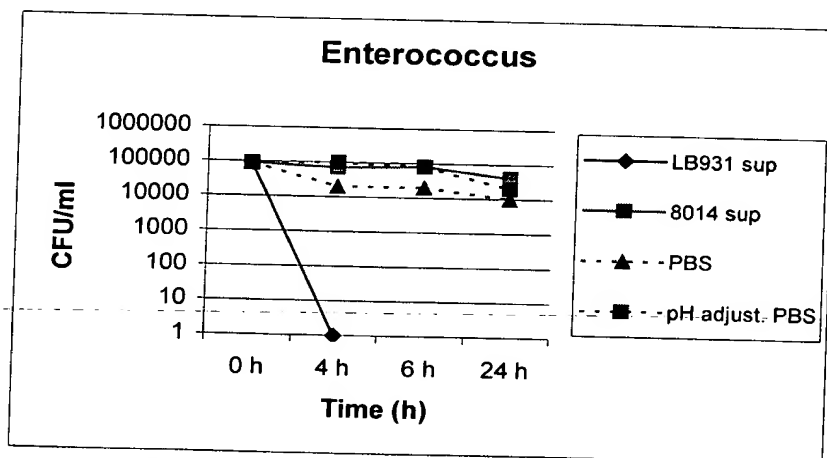


Fig. 6. Inhibition of *Enterococcus*.

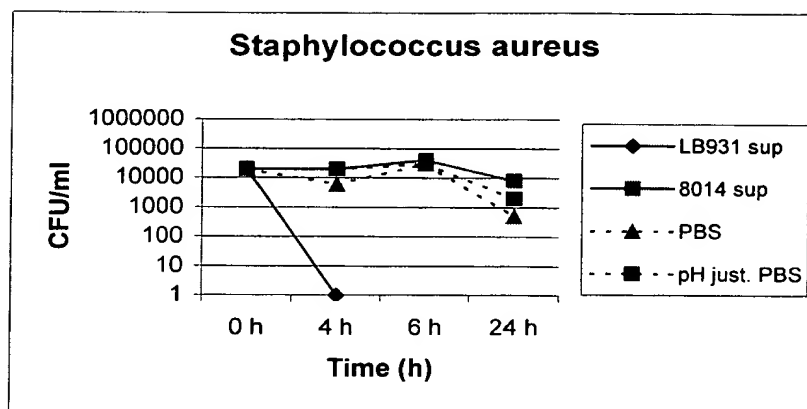


Fig. 7. Inhibition of *Staphylococcus aureus*.

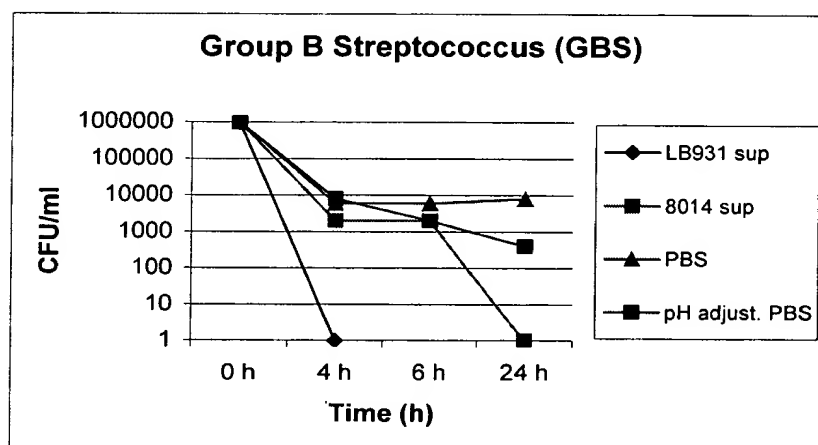


Fig. 8. Inhibition of Group B *Streptococcus* (GBS).

Conclusions

As is illustrated in the amended experimental results, the ability of LB931 to be transferred and maintained in the urogenital area is higher than it is for another *L. plantarum* strain, namely ATCC 8014. Also there is a very pronounced difference between the two *L. plantarum* strains in their ability to inhibit growth of and killing pathogenic bacteria, with LB931 being superior over ATCC 8014. Therefore there is a clear advantage in using LB931 as a probiotic bacterium in hygiene products over other *Lactobacillus plantarum* strains.

I would also like to mention that I was unable to obtain *L. plantarum* ATCC 55883, since the strain was not available from ATCC due to reasons beyond my control. Accordingly it has not been possible to perform comparative experiments using this strain.

I declare that all statements made herein are from our own knowledge and experience and are believed to be true. I am aware that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Umeå 17 may 2002

A handwritten signature in black ink, appearing to read 'Maria Isaksson', with a long horizontal flourish extending to the right.

Maria Isaksson

PATENTS

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Eva GRAHN HÅKANSSON et al.

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Filed November 16, 2000

Examiner S. Saucier

TECH CENTER 1600/2900

AGENT

DECLARATION OF AVAILABILITY AND MAINTENANCE OF MICRORGANISM

Commissioner for Patents

Washington, D.C. 20231

Sir:

SCA Hygiene Products AB, assignee of the above-identified application, declares that DSM11918, deposited on or about 9 January 1998 in the permanent collection at the Deutsche Sammlung von Mikroorganismen, Braunschweig, DE, will be available during the pendency of the above-identified application to one determined by the Commissioner of Patents and Trademarks to be entitled to access thereto under Rule 14 of the Rules of Practice in Patent Cases and 35 USC 122, and that all restrictions on the availability to the public of the culture so deposited will be irrevocably removed upon the granting of the patent; that the deposit will be replaced should the culture die during the pendency of the application or during the life of the patent, and that whatever steps will be taken that are needed in order to maintain the culture in its above-described accessibility and availability for 30 years in accordance with the Budapest Treaty or for five years after the last request for the deposit at the

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depository or for the enforceable life of the U.S. patent to issue, whichever is longer.

Bengt Forshult declares that he/she is Patent Manager of the assignee company and is authorized to sign this declaration on behalf of the company. The undersigned declares further that all statements made herein of his/her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SCA Hygiene Products AB

By

Bengt Forshult

Date 3 April 2002



In partnership with **ATCC™**

STATEMENT

ATCC 55883 *Lactobacillus plantarum*

ATCC 55883 *Lactobacillus plantarum* is a restricted patent item, on regulatory hold. It is not available for sale from ATCC at this time.

Restricted patent items is not characterized by the ATCC (ATCC has not done any major characterization, verification or amplification of this material).

On behalf of
ATCC professional Service Team

A handwritten signature in black ink, appearing to read 'B. Norlie'.

Bente Norlie
ATCC project manager
LGC Promochem AB

Borås, 2002-06-06